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Thermosensitive Ion Channel Activation in Single Neuronal Cells by Using Surface-Engineered Plasmonic Nanoparticles**

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Abstract: Controlling cell functions using external photo-responsive nanomaterials has enormous potential for the development of cell engineering technologies and intractable disease therapies, but the former currently requires genetic modification of the target cells. Here, we present a method using plasma membrane-targeted gold nanorods (pm-AuNRs) prepared with a cationic protein/lipid complex to activate a thermosensitive cation channel, TRPV1, in intact neuronal cells. Highly localized photothermal heat generation mediated by pm-AuNRs induced Ca^{2+} influx solely via TRPV1 activation. In contrast, whereas previously reported cationic AuNRs, coated with a conventional synthetic polymer, also yielded photoinduced Ca^{2+} influx, but this influx resulted from membrane damage. Our method provides an optogenetic platform without the need for prior genetic engineering of the target cells and might be useful for novel TRPV1-targeted phototherapeutics.

Photocontrol of endogenous neuronal cells could provide an appealing optogenetic methodology; for example, such localized stimulation by near-infrared (NIR) illumination might potentially be utilized as a non-invasive therapeutic modality.[1] However, current optogenetic approaches generally require prior genetic modification of the target cells,[2] which limits their broad application. The transient receptor potential vanilloid type 1 (TRPV1) is a Ca^{2+}-permeable polymodal channel gated by noxious physical and chemical stimuli including heat (> 43°C), low pH (<5.2), capsaicin, and *Euphorbia* toxin resiniferatoxin.[3] TRPV1 is present in both the peripheral and central nervous systems; in the latter, its expression is restricted to small and medium sized dorsal root ganglion (DRG) neurons. TRPV1 might serve as a therapeutic target in DRG neurons[4] and in other cells;[5] however, localized stimulation of TRPV1 in neurons by external stimuli has not yet been demonstrated. Here, we sought to develop a method that provides safe photothermal heating of TRPV1 on the surface of a single intact neuron by NIR light.

Localized heating with plasmonic nanoparticles is an emerging technology for lipid bilayers. The photoinduced generation of transient nanopores has been shown to allow the flow of biological substances into live cells[6] and artificial lipid bilayers.[7] These results motivated us to propose a more sophisticated plasma membrane (PM) heating system relevant to optogenetics as well as to phototherapies, i.e., the photoactivation of TRPV1 with gold nanorods (AuNRs), which are plasmonic nanoparticles that absorb minimally-invasive NIR light to generate heat,[8] because we previously reported that AuNRs coated with a genetically cationized form of high-density lipoprotein (HDL) were able to bind to cells with very high efficiency and without cytotoxicity.[9] This procedure was shown to be applicable not only to AuNRs but also to other metal nanoparticles with different shapes and compositions. Like HDL, the mutated HDL also had a discoidal structure indicating the existence of a neutral phospholipid bilayer circumscribed with cationic peptide-fused lipid-binding protein(s). We concluded that upon binding of the mutated HDLs to AuNRs, their discoidal membranes fused with each other to yield a smooth organic layer with a thickness comparable to that of phospholipid bilayers.

Utilization of electrostatic interaction would be the simplest strategy to target AuNRs to the PM. As-synthesized AuNRs stabilized with the cationic dispersant cetyltrimethylammonium bromide interact well with cells. However, the enhanced cell interaction of such cationic nanoparticles is often accompanied by compromised membrane integrity[10] that could lead to permeabilization and associated cytotoxicity, which are counterindicated for effective membrane channel photocontrol. Therefore, the choice of an appropriate dispersant to stabilize and cationize AuNRs is crucial. In this study, we employed a further cationized form of HDL (catHDL, see Materials and Methods) as a dispersant and compared its effects with those of the well-known cationic synthetic polymers poly(diallyldimethyl) ammonium chloride (PDDAC), polyethyleneimine (PEI), and poly-L-lysine (PLL).

AuNRs with a plasmonic absorption maximum at 785 nm were coated with catHDL according to our previous report or with PDDAC, PEI, or PLL by a layer-by-layer electrostatic assembly method,[11] which stabilized the AuNRs to varying degrees (Figure 1a). The presence of catHDL and the synthetic cationic polymers on the AuNR surface was confirmed by Zeta potential and IR spectroscopy analyses (Figures 1b and S1). PM binding of the cationized AuNRs was examined by observing their two-

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photon luminescence (TPL) (Figure 1c) because the TPL intensity provides an indication of AuNR colloidal stability on the PM.[12] Only catHDL-treated AuNRs (pm-AuNRs) were found to bind adequately to HEK293T PMs following 10 min AuNR treatment (Abs780 = 0.15) (Figure 1d). Additionally, pm-AuNRs had the weakest cytotoxicity (Figure 1e), although the degree of cationization was similar for all samples (Figure 1b); the pm-AuNRs phospholipid bilayer might reduce cationic polymer-induced membrane disruption and the protein moiety could enhance pm-AuNR colloidal stability even on the PM. Accordingly, we decided to further pursue pm-AuNRs.

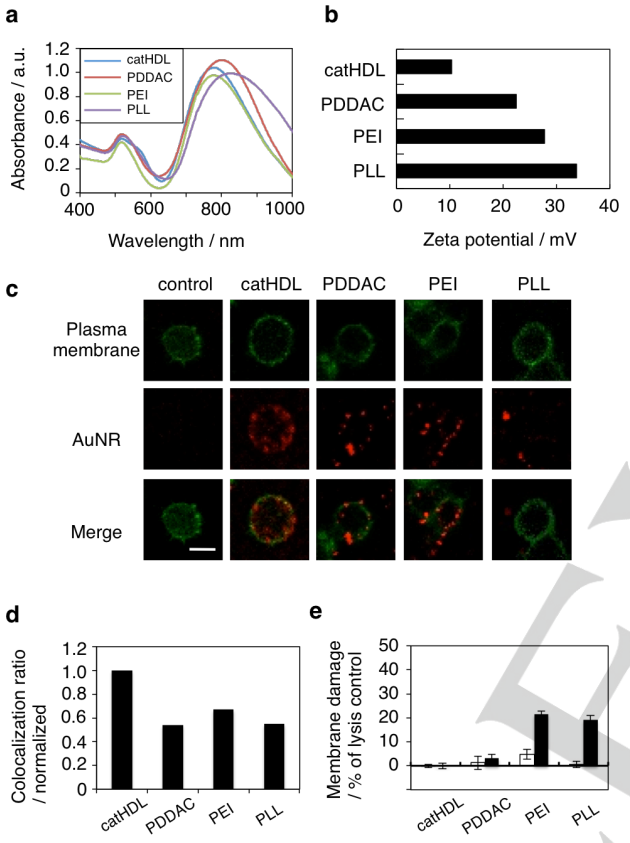

Figure 1. Characterization of surface-functionalized AuNRs. a) Vis-NIR absorption spectra of various AuNRs in PBS. Various peak wavelengths are seen, probably due to local dielectric function of the dispersants and/or different colloidal stabilities.[13] b) Zeta potential data of the AuNRs. All AuNRs have positive values. c) Two-photon luminescence microscopy images of HEK293T cells treated with various AuNRs (Abs780 = 0.15, 10 min). The signals derived from AuNRs and pEGFP-f staining of the plasma membrane (PM) are shown in red and green, respectively. Only pm-AuNRs appear to colocalize well with the PM. Bar, 10 μm. d) Colocalization ratios of AuNR and EGFP fluorescence. The colocalization ratio is defined as the number of red pixels colocalizing with green pixels in cells/total red pixels in cells. Higher ratios indicate greater AuNRs binding to the PM. It can be seen that pm-AuNRs exhibit the most effective binding to the PM among the varieties tested. e) Lactate dehydrogenase assay data for cells treated with various AuNRs at Abs780 = 0.15 (white bar) or 0.3 (black bar) for 10 min. Data are normalized to both non-treated (0% membrane damage) and 0.02% Tween 20-treated (100% membrane damage) cells. pm-AuNRs demonstrate the weakest cytotoxicity among the AuNRs examined.

TRPV1 photoactivation by pm-AuNRs (Figure 2a) was evaluated in a Ca^{2+} flux assay based on fluorescence detection of a Ca^{2+} indicator, Fluo3-AM, using an epifluorescence microscope equipped with a wavelength-tunable laser (715–950 nm) (Figure S2). Upon illumination at 780 nm (8 μW/μm^2) of a single TRPV1-overexpressing HEK293T cell bearing pm-AuNRs on the PM, the fluorescence intensity was observed to increase (Figure 2b) and the degree of this increase depended upon the illumination time (Figure 2c). This Ca^{2+} influx was not detected in the absence of either TRPV1 expression (Figure 2d), pm-AuNR treatment (Figure S3a), or illumination (Figure S3b), and was suppressed by half at a lower incubation temperature (Figure S3c). These results, summarized in Figure 2e, unambiguously corroborated that PM-bound AuNRs photoactivated TRPV1 via photothermal heat generation without membrane disruption.

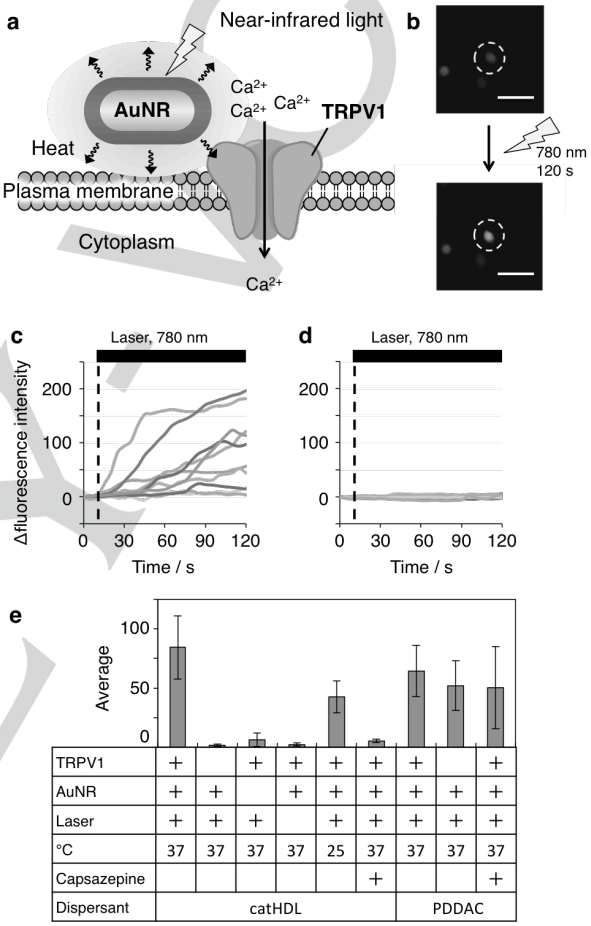

Figure 2. Photoactivation of TRPV1-overexpressing HEK293T cells by AuNRs. a) Schematic of localized photothermal heating of a TRPV1-expressing PM bearing pm-AuNRs. Transduced cells were loaded with an intracellular Ca^{2+} indicator, Fluo 3-AM (Dojindo), treated with pm-AuNRs (Abs780 = 0.15), and then illuminated at 780 nm (8 μW/μm^2) under incubation at 37°C. b) Fluorescence images of cells before and after illumination. Dotted circles indicate the illuminated area. Bar, 100 μm. Time-dependent change in the fluorescence intensities of c) transduced and d) intact cells under illumination. Each curve was obtained from a single cell. The average and standard deviation values for the maximal fluorescence intensities during 120 s illumination in various conditions are summarized in e). The TRPV1-dependency of the photoactivation was further examined by adding a TRPV1 antagonist, capsazepine. Capsazepine treatment completely inhibits Ca^{2+} influx induced by pm-AuNRs under illumination. In contrast, AuNRs cationized with PDDAC are not able to induce TRPV1-dependent Ca^{2+} influx.

We next evaluated the potential adverse effects of this photoactivation. Addition of capsazepine, a TRPV1 antagonist, throughout (Figures S3d and 2e) or halfway through (Figure S3e) photoactivation almost completely blocked Ca^{2+} influx,

suggesting that localized heat generation evinced negligible detrimental effects on TRPV1. As shown in Figure S4, no indications of necrosis or apoptosis were detected after photoactivation.

PDDAC-stabilized AuNRs, which showed the least dark cytotoxicity among the cationic polymer-coated AuNRs examined (Figure 1e), also yielded a photoinduced Ca^{2+} influx, but this was independent of both TRPV1 expression and function (Figure 2e) and laser power intensity (Figure S5), suggesting that this effect was derived from membrane disruption potentially from less-uniform heat generation (Figure 1c) and associated damage rather than TRPV1 activation.[14] Similarly, it has been reported that the photodynamic and photothermal effects of photosensitizer-loaded carbon nanoparticles could additively induce Ca^{2+} influx in various types of cells including macrophages by unknown mechanisms, for which the possibility of involvement of membrane disruption is not excluded.[15] In contrast to the results of this work, our results demonstrate that pm-AuNRs enable safe activation of a defined single type of ion channel solely via localized photothermal heating of the PM, thus highlighting the importance of AuNR surface chemistry.

To confirm whether photoactivation was caused by a local temperature rise, the temperature dependences of the fluorescence intensities[16] of pm-AuNR-incorporated 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N -(lissamine rhodamine B sulfonyl (Rho-PE) and LysoTracker Green DND-26 stained late endosomes/lysosomes were used as thermometers (Figures S6 and S7a). Upon laser illumination, the temperature of the PM reached over 43°C within 120 s on average, whereas the temperature of the late endosomes/lysosomes was unchanged (Figure S7b). These data also exemplify that the photothermal heating by pm-AuNRs is the trigger to activate TRPV1 and is confined to the immediate PM vicinity.

We also compared the photoactivation efficiencies of pm-AuNRs at various laser wavelengths around the absorption peak (780 nm) to verify the importance of plasmon resonance absorption. We found that the relative Ca^{2+} influx was increased only at 780 nm or shorter and was greatly suppressed at the other tested wavelengths (Figure 3a-e). These results seemed to contradict the plasmon absorption profile of pm-AuNRs, which showed consistent, lower levels of suppression extending outward from the absorption peak (Figure 1a), because the degree of temperature elevation for AuNR dispersion illuminated at each wavelength was entirely dependent on the absorbance of the pm-AuNRs (Figure S8). Cell–nanoparticle interaction has been reported to be affected by the size and shape of nanoparticles,[17] which greatly affect the plasmon resonance absorption of AuNRs. To gain insight into the cell-adsorbed fractions of pm-AuNRs, a differential absorption spectrum was obtained by subtracting the spectrum of the cell supernatant from that of the original pm-AuNRs; consequently, the absorption maximum was found to be blue-shifted from 780 to 745 nm (Figure 3f) and the actual respective absorbance values were estimated to be ordered as 735 > 780 > 715 > 830 > 855 nm in accordance with the fluorescence intensity data summarized in Figure 3f. Furthermore, we found that photoinduced TRPV1-dependent Ca^{2+} influx became possible even at 812 nm, with half the absorbance at 780 nm, when the laser power intensity was doubled (Figure S9). These results emphasize the importance of the plasmon resonance of AuNRs on TRPV1 photoactivation.

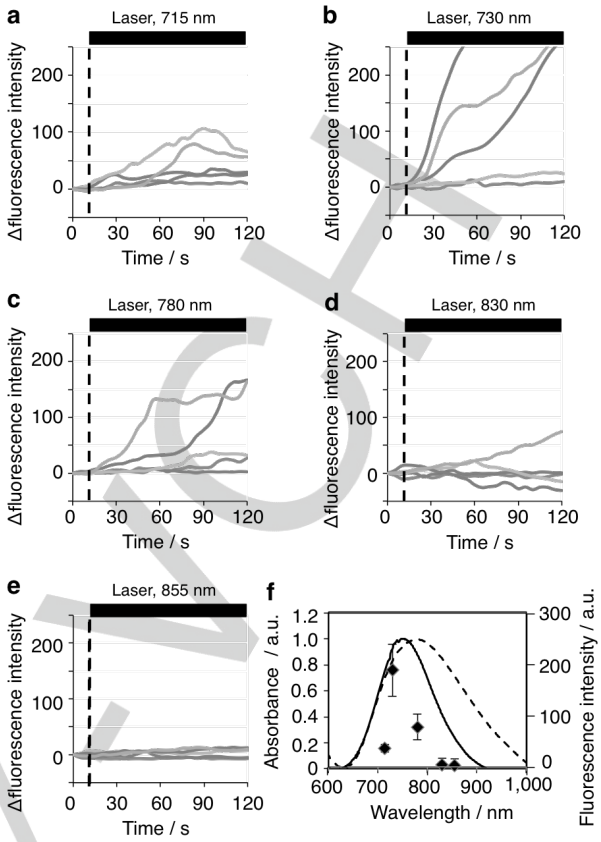

Figure 3. Plasmonic enhancement of TRPV1 photoactivation. TRPV1-overexpressing HEK293T cells bearing pm-AuNRs were illuminated at a) 715, b) 735, c) 780, d) 830, and e) 855 nm. Compared to the absorbance at 780 nm (reflecting the plasmon absorption maximum of pm-AuNRs), the absorbance values at 715 and 855 nm and at 730 and 830 nm were 60% and 80%, respectively (Figure 1a). f) The Vis-NIR absorption spectrum of pm-AuNRs in HBSS before cell treatment (dashed curve) and cell adsorbed fraction of pm-AuNRs (solid curve). The latter is the differential absorption spectrum obtained by subtracting the spectrum of the cell supernatant containing unbound pm-AuNRs from the spectrum of pm-AuNRs before cell treatment. In f), the average and standard deviation data for the maximal fluorescence intensity during 120 s illumination for 10 cells in a-e) are also shown as a scatter diagram. The plasmon absorption peak of the cell-adsorbed fraction of pm-AuNRs can be seen to be blue-shifted from 780 nm to 745 nm, and this plasmon absorption profile is in accordance with the data shown in the scatter diagram. This plasmon resonance blue shift indicates a preferential binding of the lower aspect-ratio fractions of pm-AuNRs.[8]

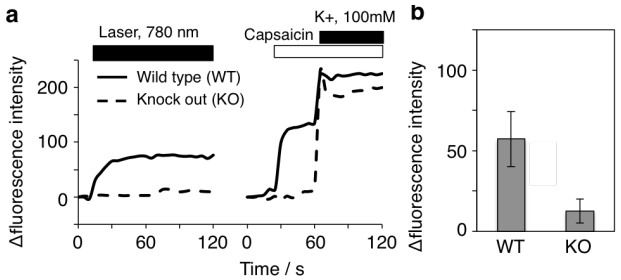

Figure 4. Photoactivation of primary cultured neuronal cells by AuNRs. a) Ca^{2+} influx photoinduced in dorsal root ganglion (DRG) neurons of wild-type (WT) and TRPV1 knock out (KO) mice. Representative Ca^{2+} responses in WT (solid line) and KO (broken line) neurons under the same conditions as in Figure 2 are shown. Once the Ca^{2+} concentration in the illuminated WT cells had returned to the basal level, we added capsaicin (1 μM), a TRPV1 agonist, followed by 100 mM K^+ solution to induce depolarization to evaluate TRPV1 expression and neuronal activity of the illuminated cells, respectively. b) Average and standard deviation values of five cells for maximal fluorescence

intensities during 120 s illumination. Five of 10 capsaicin-responsive WT cells were photoactivated by AuNRs.

Finally, the feasibility of our method for TRPV1 photoactivation under nearly physiological conditions was assessed in primary-cultured DRG neurons from wild-type mice (some of which express TRPV1) as well as from TRPV1 knockout (KO) mice. As shown in Figure 4, pm-AuNRs achieved photoinduced Ca^{2+} influx in primary cultured DRGs from wild-type but not from TRPV1 KO mice. Maintenance of illuminated cell neuronal activity was confirmed by observing the depolarization induced with high K+ (Figure 4a). These results support that our method safely photoactivates TRPV1 under physiological conditions.

Magnetic nanoparticles have also been shown to be able to activate overexpressed TRPV1 channels in HEK293/T cells through localized heating stimulated by the application of a radiofrequency magnetic field.[16a, 18] However, although the use of magnetics might allow deep tissue penetration, this strategy required genetic modification of cells as well as over 1,000 times the nanoparticle concentration to achieve a similar degree of heating as that achieved with plasmonic nanoparticles.[9, 18] Thus, plasmonic nanoparticles currently have at least 1,000 times greater heating power than magnetic nanoparticles. Additionally, light provides a more precisely localized stimulation, and the increased rate of photothermal heating compared to that of magnetic field-induced heating is also advantageous.[19]

Clinically, TRPV1 is a potential therapeutic target for nociceptive pain and cancers.[4-5] Lack of requirement of prior genetic engineering of the target cells for TRPV1 activation under minimally invasive NIR illumination is one of the novelties of our method. Furthermore, compared with small molecules, nanomaterials are retained in the body for a prolonged period; therefore, local injection of pm-AuNRs might enable repetitive and on-demand treatment of these disorders.

In conclusion, we have developed an unprecedented optogenetic means to generate thermosensitive cation channel activation in the PM of intact cells using localized photothermal heating of NIR-plasmonic nanoparticles. This success was dependent upon modulation of the mesoscopic surface chemistry of the nanoparticles using a cationic lipoprotein, which is an indispensable factor for their uniform and noncytotoxic PM delivery through suppression of the PM disruption associated with photothermal heating. Additionally, from the point of view of future medical applications, the utilization of the human protein-based material should be beneficial. Our strategy for mesoscopic surface chemistry could also potentially enable the localized photothermal heating of specific intracellular organelles while minimizing thermal membrane damage, opening up novel areas of research.

Keywords: surface chemistry • gold nanorods • ion channel • photothermal effect • cell engineering

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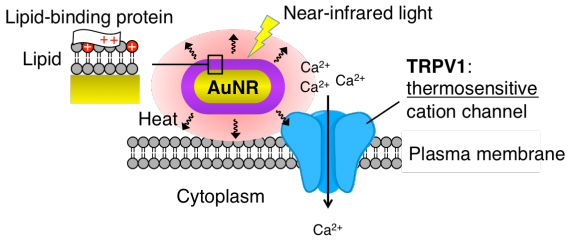

Non-disruptive plasma membrane heating technology was developed by using gold nanorods (AuNRs) coated with a cationic protein/lipid complex. Under near-infrared illumination, this AuNRs were capable of highly localized photothermal heat generation in intact neuronal cells without membrane damage, inducing Ca^{2+} influx solely via activation of a thermosensitive cation channel, TRPV1. This success was dependent upon modulation of the surface chemistry of AuNRs.

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